Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 6190–6194, June 1995
Neurobiology

Insulin-like growth factor I treatment reduces demyelination and up-regulates gene expression of myelin-related proteins in experimental autoimmune encephalomyelitis

(oligodendroglia/myelin basic protein/proteolipid protein/myelin regeneration/multiple sclerosis)

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Communicated by Richard L. Sidman, New England Regional Primate Research Center, Southborough, MA, March 20, 1995

ABSTRACT **To compare effects of insulin-like growth factor I (IGF-I) and placebo treatment on lesions that resemble those seen during active demyelination in multiple sclerosis, we induced experimental autoimmune encephalomyelitis in Lewis rats with an emulsion containing guinea pig spinal cord and Freund's adjuvant. On day 12–13, pairs of rats with the same degree of weakness were given either IGF-I or placebo intravenously twice daily for 8 days. After 8 days of placebo or IGF-I (200 μg/day or 1 mg/day) treatment, the spinal cord lesions were studied by *in situ* hybridization and with immunocytochemical and morphological methods. IGF-I produced significant reductions in numbers and areas of demyelinating lesions. These lesions contained axons surrounded by regenerating myelin segments instead of demyelinated axons seen in the placebo-treated rats. Relative mRNA levels for myelin basic protein, proteolipid protein (PLP), and 2′,3′-cyclic nucleotide 3′-phosphodiesterase in lesions of IGF-I-treated rats were significantly higher than they were in placebo-treated rats. PLP mRNA-containing oligodendroglia also were more numerous and relative PLP mRNA levels per oligodendrocyte were higher in lesions of IGF-I-treated rats. Finally, a significantly higher proportion of proliferating cells were oligodendroglia-like cells in lesions of IGF-I-treated rats. We think that IGF-I effects on oligodendrocytes, myelin protein synthesis, and myelin regeneration reduced lesion severity and promoted clinical recovery in this experimental autoimmune encephalomyelitis model. These IGF-I actions may also benefit patients with multiple sclerosis.**

When experimental autoimmune encephalomyelitis (EAE) is induced in Lewis rats with an emulsion containing guinea pig spinal cord and Freund's adjuvant, the spinal cord lesions resemble those seen during active demyelination in multiple sclerosis (1–4). About 12 days after EAE induction, the blood–spinal cord barrier is permeable to macromolecules and there are prominent demyelinating lesions associated with inflammation and relative sparing of axons (3, 5). Since similar changes are also found in actively demyelinating multiple sclerosis lesions, we selected this EAE model to test the possible usefulness of growth factor treatment for multiple sclerosis.

Several lines of evidence suggest that insulin-like growth factor I (IGF-I) might be useful in reducing myelin breakdown and promoting myelin regeneration in demyelinating diseases. IGF-I promotes the survival of oligodendroglia and the formation of myelin sheaths *in vitro* (6–8). The content of myelin also is increased in the central nervous system of transgenic mice that overexpress IGF-I (9). Reactive astrocytes synthesize high levels of IGF-I in three *in vivo* models of demyelination in which oligodendroglia express the receptor for IGF-I while myelin sheaths regenerate. These models are acute demyelinating EAE (3), cryogenic spinal cord injury (10), and cuprizone-induced demyelination (11). Finally, IGF-I treatment has been shown to reduce clinical deficits, blood–spinal cord permeability, and lesion severity in acute demyelinating EAE (5).

Our goal here was to compare severity of demyelination, gene expression of myelin-related proteins, and oligodendrocyte proliferation in EAE lesions of rats treated with either IGF-I or placebo. We discovered that IGF-I treatment reduced demyelination and increased oligodendroglial gene expression of myelin-related proteins significantly. IGF-I also increased proliferation of oligodendroglia-like cells and promoted myelin regeneration.

MATERIALS AND METHODS

Animals and Experimental Design. For the first IGF-I treatment trial, EAE was induced in 30 anesthetized male Lewis rats (250–300 g) as described (3). On day 12, when mild but definite weakness was first detected, 8 pairs of rats with identical clinical deficits were identified. Eight rats were given 100 μg of recombinant human IGF-I (a gift of Genentech) intravenously in the tail vein every 12 hr for 8 days. The other 8 rats received intravenous placebo injections (0.89% NaCl). On day 20, after 8 days of IGF-I or placebo treatment, 5 pairs of rats were anesthetized and perfused with an aldehyde solution before removing their spinal cords. After anesthesia, spinal cords from the remaining 3 pairs of rats were removed rapidly and frozen. Spinal cord segments from all 16 rats were processed according to previously described methods for *in situ* hybridization, immunocytochemistry, or morphological study of stained paraffin or semithin epoxy resin sections (3, 12, 13).

EAE was induced in 70 more rats in order to select 11 pairs (22/40) for the second and 6 pairs (12/30) for the third treatment trials. The procedures were the same except for the IGF-I dose; it was 500 instead of 100 μg twice daily. All procedures met National Institutes of Health guidelines for use of animals in research.

Nucleotide Probes and *in Situ* Hybridization Procedures. Synthetic oligonucleotide probes were used that are specific for myelin basic protein (MBP), proteolipid protein (PLP), and 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNP). The radiolabeled probes, their preparation, as well as the methods used for *in situ* hybridization and the analysis of x-ray autoradiograms have been described (12–15). Mean densities (aver-

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Abbreviations: IGF-I, insulin-like growth factor I; EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; PLP, proteolipid protein; CNP, 2′,3′-cyclic nucleotide 3′-phosphodiesterase; MOG, myelin-oligodendrocyte glycoprotein.

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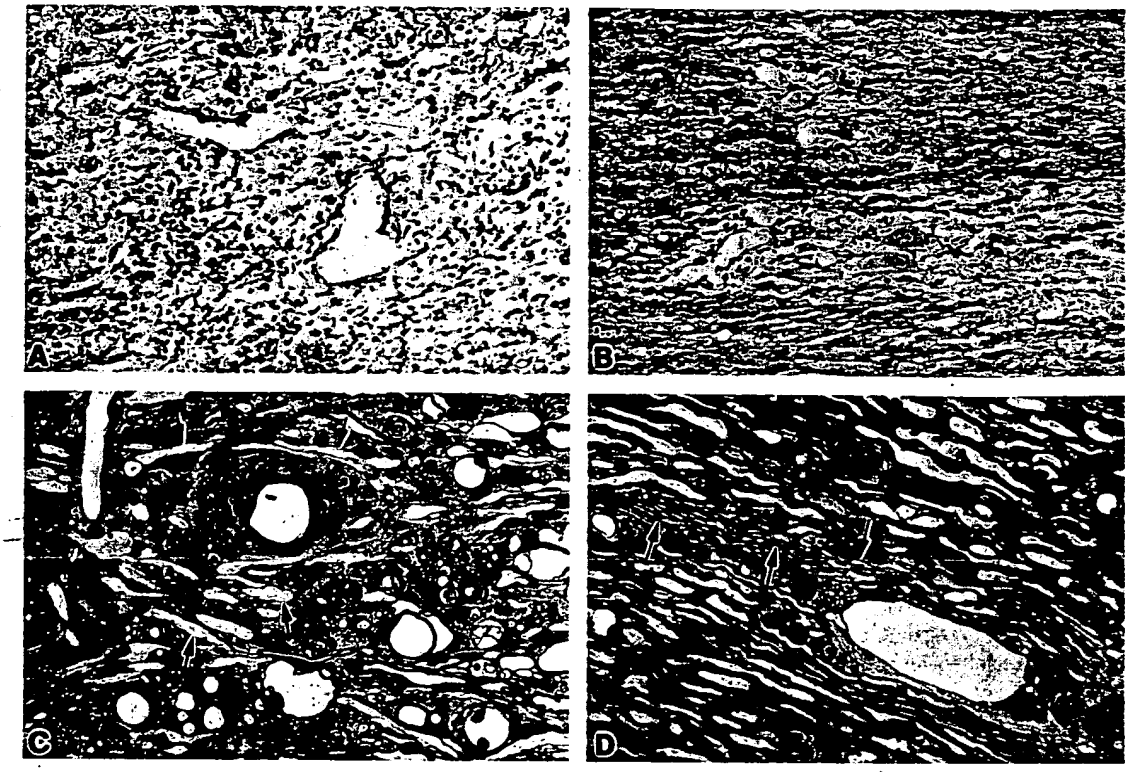

FIG. 1. Spinal cord sections from placebo-treated rats contain larger areas of demyelination, more inflammation (*A*), and more demyelinated axons (arrows in *C*) than those from IGF-I-treated rats (*B* and *D*). Thin, short myelin segments (arrows) surround axons in *D*. [Anti-MBP immunostaining (*A* and *B*), ×380; toluidine blue-stained semithin sections (*C* and *D*), ×600.]

age grey level of the pixels within a given area) were measured in arbitrary units in comparable areas of eight nonoverlapping longitudinal spinal cord sections from 3 pairs of rats and were expressed as percentages of densities in the same areas in normal rats ± SE. For microscopic analysis of emulsion autoradiograms, we used Bioquant OS/2 software to count the number of PLP probe-containing oligodendrocytes per lesion and also to determine the grain density of the hybridized PLP probe per lesion oligodendrocyte. Means ± SE of these values were determined for all lesions found in four nonoverlapping longitudinal spinal cord sections from 3 pairs of rats in each dosage group.

Immunochemicals and Immunocytochemical Procedures. Anti-MBP, anti-transferrin, and anti-BrdUrd were purchased from Dako. Other antibodies were anti-PLP (Agmed, Bedford, MA), anti-galactocerebroside (Boehringer Mannheim), anti-CNP (gift of P. Braun, McGill University, Montreal), and monoclonal anti-myelin-oligodendrocyte glycoprotein (MOG) (gift of C. Linnington, Max-Planck-Institut for Psychiatrie, Martinsried, Germany). Immunostaining was done according to the avidin-biotin peroxidase complex (ABC) method (16) using biotinylated secondary antibodies (Vector Laboratories) and previously described methods (10, 13).

Combined *in Situ* Hybridization and Immunocytochemistry. Unfixed frozen longitudinal spinal cord sections were hybridized *in situ* with oligonucleotide probes for MBP, PLP, or CNP before immunostaining them with anti-BrdUrd or antibodies used to immunolabel oligodendrocyte—namely, anti-MOG, anti-CNP, anti-galactocerebroside, and anti-transferrin (10).

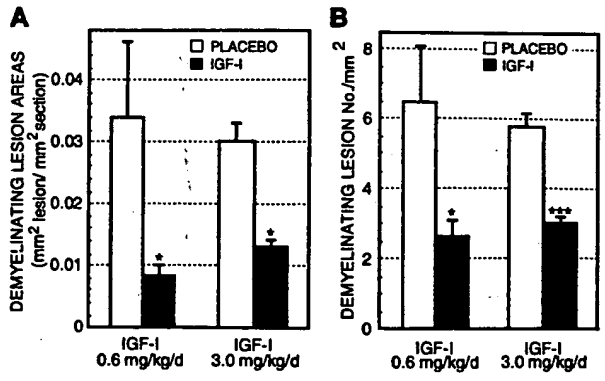

FIG. 2. After 8 days of IGF-I treatment, areas of demyelinating lesions are smaller (*A*; *, $P \leq 0.05$; $n = 10$) and there are significantly fewer lesions (*B*; *, $P \leq 0.05$; ***, $P \leq 0.0001$; $n = 10$) than after placebo treatment.

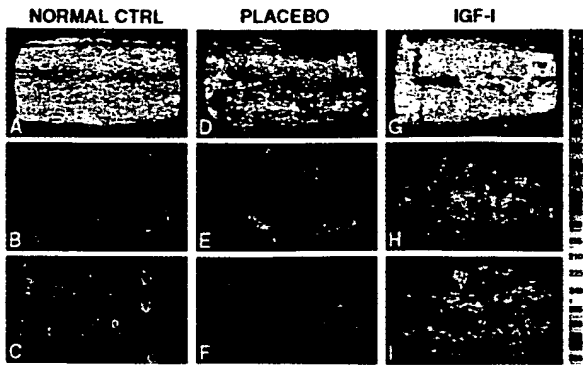

FIG. 3. Computer-generated pseudocolor images of relative mRNA levels of MBP (*A, D,* and *G*), PLP (*B, E,* and *H*) and CNP (*C, F,* and *I*) in longitudinal spinal cord sections of normal adult rats (*A–C*) and of rats treated for 8 days with placebo (*D–F*) or with IGF-I, 3 mg/kg per day (*G–I*). Levels are highest (orange-red) in and around lesion areas of IGF-I-treated rats. Compared to those treated with IGF-I, placebo-treated rats have lower relative levels (*D–F*), and relative levels for MBP (*D*) and CNP (*F*) are below those seen in normal rats.

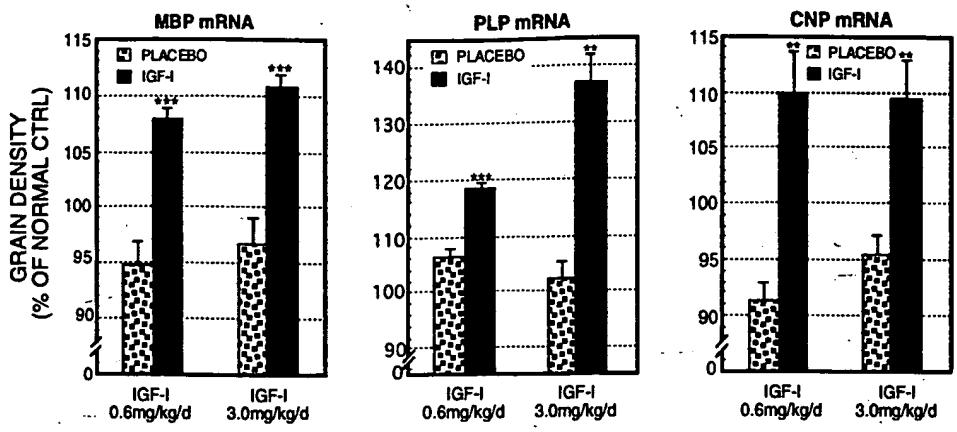

FIG. 4. Relative mRNA levels for MBP, PLP, and CNP are significantly higher in rats treated for 8 days with both doses of IGF-I (**, $P \leq 0.01$; ***, $P \leq 0.0001$; $n = 6$) than they are in placebo-treated rats.

Rats in the second and third treatment trials were injected with BrdUrd before sacrifice; sections were immunostained with anti-BrdUrd and counterstained with hematoxylin. After covering slide labels of four nonoverlapping spinal cord sections from each of 8 pairs of rats, diameters of 450 BrdUrd- and non-BrdUrd-labeled nuclei in lesions were measured. Lymphocytes and other mononuclear inflammatory cells had small nuclei (6.25 ± 0.33 μm); oligodendroglial and astroglial nuclei were larger (8.33 ± 0.24 μm).

RESULTS

Morphology. In paraffin and semithin sections of placebo-treated rats, the demyelinating lesions with inflammation and intact axons resembled those described earlier (3) (Fig. 1*A* and *C*). However, in sections of spinal cords from IGF-I-treated rats, demyelinated areas in white and grey matter were much smaller; they also contained substantially fewer inflammatory cells than those from placebo-treated rats (Fig. 1*B* vs. *A*). After placebo treatment for 8 days, large lesions in semithin sections contained many demyelinated axons (Fig. 1*C*). Similar sections from IGF-I-treated rats contained much smaller lesions that had almost no demyelinated axons (Fig. 1*D*). Instead, axons were surrounded by very thin, short myelin segments, an appearance consistent with myelin regeneration (Fig. 1*D*).

After covering labels of anti-MBP-stained sections and randomizing them, numbers of demyelinating lesions were counted and their areas were measured. In sections from rats treated for 8 days with both doses of IGF-I, there were 50–70% fewer lesions and they were about 50–75% smaller; both differences were significant (Fig. 2).

Myelin-Related Protein mRNAs. Computerized analysis of relative grain densities on x-ray films provided semiquantitative comparisons of relative mRNA levels for MBP, PLP, and CNP. All of these levels were significantly elevated in sections of IGF-I-treated rats (Figs. 3 and 4). Furthermore, the IGF-I-induced increases were concentrated in and around lesions (Fig. 3).

After 8 days of IGF-I treatment, MBP mRNA levels were 13–16% above those observed after placebo treatment (Figs. 3 and 4). When emulsion autoradiograms of sections from placebo- and IGF-I-treated rats were compared, there also were striking differences in MBP mRNA levels and distributions. At low magnification (Fig. 5 *A* and *B*), grain densities in lesions of placebo-treated rats were much lower than in surrounding white matter (Fig. 5*A*). However, in IGF-I-treated rats, lesion levels of MBP mRNA were much higher than in

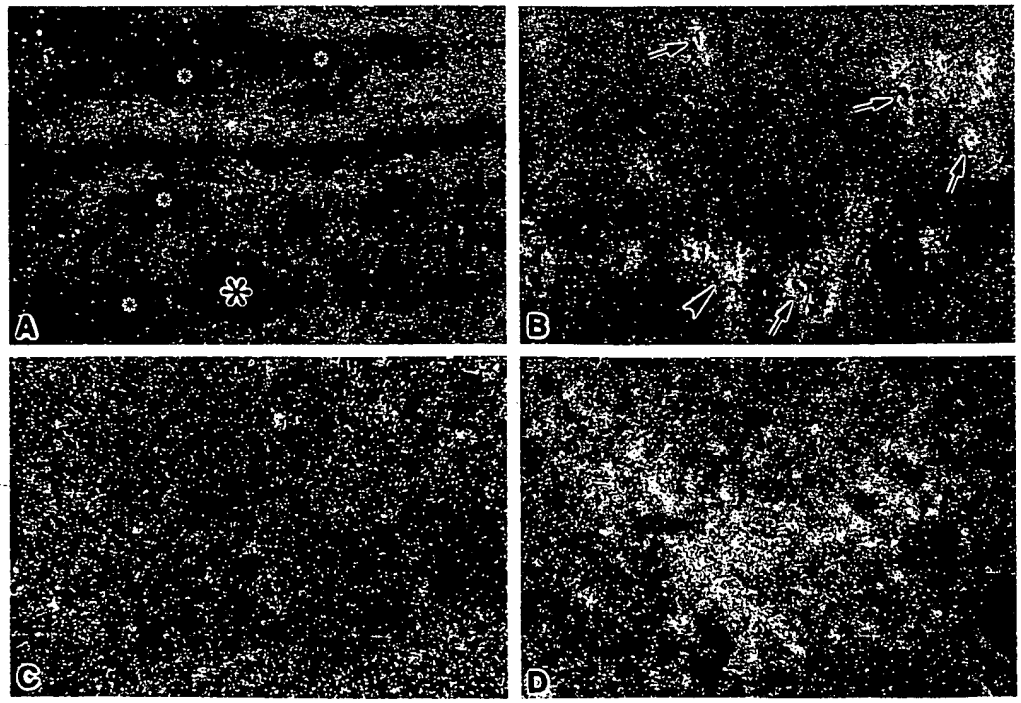

FIG. 5. Emulsion autoradiograms of MBP mRNA distribution after 8 days of placebo (*A* and *C*) or 0.6 mg of IGF-I per kg per day (*B* and *D*). In *A* and *C*, MBP mRNA levels are significantly reduced in lesion areas (* in *A*; *C* is area of large *) of placebo-treated rats. Grain densities, reflecting MBP mRNA levels, are much higher in lesion areas of IGF-I-treated rats (*B*, arrows; area of lower left arrowhead in *B* is magnified in *D*). (*A* and *B*, ×48; *C* and *D*, ×190.)

non-lesion areas (Fig. 5*B*). At high magnification (Fig. 5 *C* and *D*), MBP mRNA-containing oligodendrocytes were covered by punctate areas of increased grain density. Almost no densely labeled oligodendroglia were present in lesion areas of placebo-treated rats and perilesion areas contained very few (Fig. 5*C*). In contrast, lesion areas of IGF-I-treated rats contained many oligodendrocytes with very high levels of hybridized MBP probe (Fig. 5*D*).

After 8 days of IGF-I treatment, levels of PLP mRNA were 12–34% above those observed in placebo-treated rats and the increases were significant (Figs. 3 and 4). Since there was consistent, punctate oligodendroglial localization of hybridized PLP probe in lesion areas of emulsion autoradiograms, we counted PLP mRNA-expressing oligodendrocytes in lesions and estimated relative cellular mRNA levels in lesions by counting the grains in each labeled oligodendrocyte. After IGF-I treatment, lesions contained about double the number of oligodendroglia that expressed PLP mRNA; also, levels of PLP mRNA per oligodendrocyte were approximately twice those seen in lesions of placebo-treated rats (Fig. 6). Both of these IGF-I-induced increases were significant (Fig. 6).

Oligodendrocytes and Cell Proliferation in Lesions. Approximate numbers of oligodendroglia-like cells in lesions were also compared in immunostained sections. Trials with antibodies raised against MOG, CNP, galactocerebroside, and transferrin showed that the most consistent counts of oligodendroglia-like cells in demyelinating lesions were obtained with anti-transferrin. Very few transferrin-positive cells that resembled oligodendroglia morphologically were seen in lesions from placebo-treated rats; many more were identified in lesions after IGF-I treatment (Fig. 7 *A* and *B*). In peri-lesion areas, oligodendroglia-like cells were also stained by anti-MOG, anti-CNP, and anti-galactocerebroside; they were much more numerous in sections from IGF-I-treated rats (data not shown).

Proliferating cells in lesions had nuclei immunostained by anti-BrdUrd. There were many more BrdUrd-labeled nuclei in lesions of placebo-treated rats. Almost all of the cells had small nuclei, looked like lymphocytes and mononuclear inflammatory cells, and were not labeled by the probe for PLP mRNA (Fig. 7*C*). After IGF-I treatment, the lesions contained substantially fewer BrdUrd-labeled nuclei but many of them belonged to oligodendrocyte-like, PLP mRNA-containing cells (Fig. 7*D*). Measurements showed that glial nuclei were significantly larger than lymphocyte and mononuclear cell nuclei. Percentages of proliferating inflammatory cells and glial cells in lesions were estimated by counting cells with small or large BrdUrd- and non-BrdUrd-labeled nuclei. These counts confirmed that there were almost 3-fold more proliferating cells in lesions of placebo-treated rats; however, only 2.5% were glial cells (Fig. 8). In contrast, 17% of the proliferating cells in lesions of IGF-I-treated rats were glia and the large majority resembled oligodendroglia morphologically (Fig. 8).

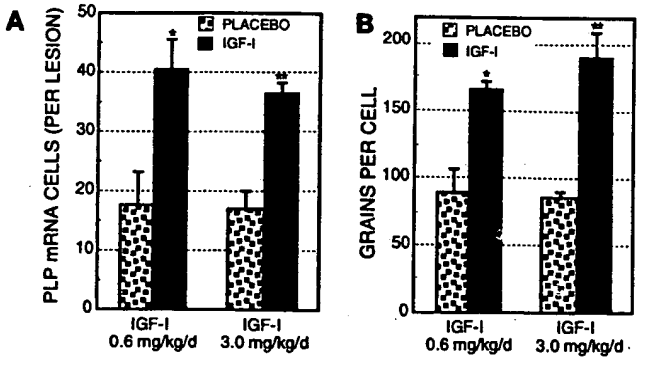

FIG. 6. There were significantly more PLP mRNA-containing cells per lesion in IGF-I-treated (*, $P \leq 0.05$; **, $P \leq 0.01$; $n = 3$) than in placebo-treated rats (*A*). (*B*) PLP mRNA levels per cell were also significantly higher after 8 days of IGF-I treatment (*, $P \leq 0.05$; **, $P \leq 0.01$; $n = 3$).

DISCUSSION

This study compares effects of IGF-I and placebo treatment on myelin and oligodendrocytes in an EAE model with lesions that resemble those seen during active demyelination in multiple sclerosis. Treatment was started 12 days after induction, when blood–spinal cord barrier changes, clinical deficits, and immune-mediated inflammatory lesions were present (5).

Our results strongly suggest that direct receptor-mediated effects of IGF-I on oligodendrocytes helped reduce demyelination and promote myelin regeneration. IGF-I crosses the blood–brain barrier, even in normal adult rats (17). IGF-I significantly increased gene expression of MBP, PLP, and CNP in and around lesions. IGF-I also produced earlier and more extensive remyelination of demyelinated axons. For PLP and MBP, mRNA levels per oligodendrocyte also were higher and lesions contained more oligodendrocytes with up-regulated mRNA expression. Increased BrdUrd labeling showed that substantially more oligodendrocyte-like cells were proliferat-

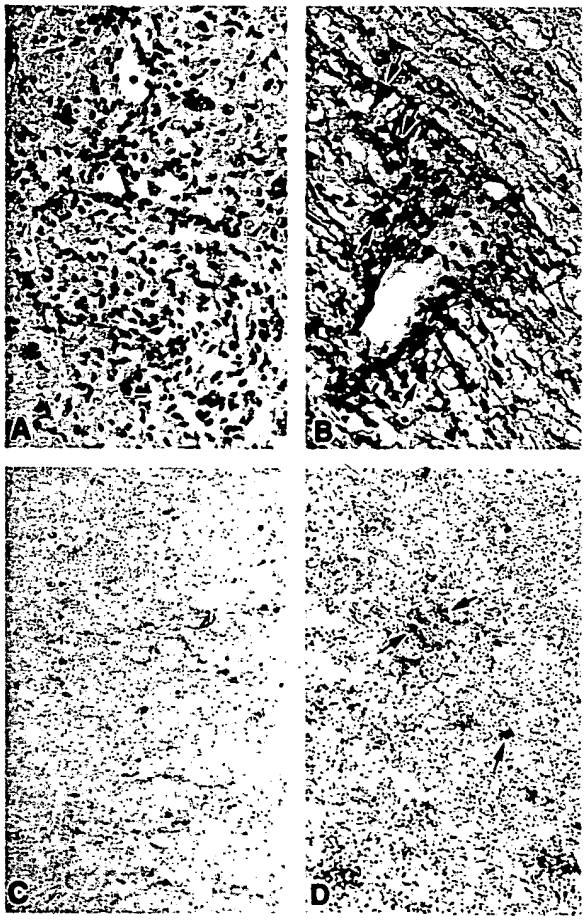

FIG. 7. Anti-transferrin immunolabeling shows immunoreactive oligodendroglia-like cells (*B*, arrows) in a lesion after 8 days of IGF-I treatment. None of these transferrin-positive, oligodendroglia-like cells are found in inflammatory lesions of placebo-treated rats (*A*). A lesion in an IGF-I-treated rat (*D*) contains oligodendroglia-like cells that are intensely labeled by the probe for PLP mRNA. The nuclei of some of these cells also are immunolabeled by anti-BrdUrd (arrows). After placebo treatment (*C*), cells with BrdUrd-immunoreactive nuclei are smaller and resemble mononuclear inflammatory cells; they are not labeled by the probe for PLP mRNA. (*A* and *B* were counterstained with hematoxylin; *A–D*, ×380.)

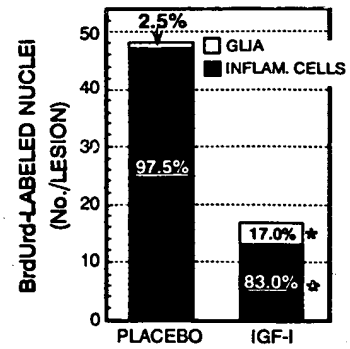

FIG. 8. Lesions of rats treated with IGF-I for 8 days contain substantially fewer BrdUrd-labeled nuclei than those of placebo-treated rats but the percentage of labeled glial nuclei is significantly higher (*, $P \leq 0.05$; $n = 8$).

ing and expressing myelin-related constituents in and around lesions of IGF-I-treated rats. These effects on oligodendroglia probably helped reduce the clinical deficits that we described earlier in this model (5).

Evidence obtained previously in tissue culture experiments and *in vivo* studies strongly supports the suggestion that direct receptor-mediated actions of IGF-I are responsible for these effects. IGF-I promotes oligodendrocyte proliferation and survival *in vitro* (7, 8, 18); it also stimulates oligodendrocyte development and formation of myelin in rat brain aggregate cultures (6). Myelin production per oligodendrocyte is increased in transgenic mice overexpressing IGF-I (9). Also, oligodendrocytes and myelin are deficient in mice homozygous for a disrupted IGF-I gene (19). Finally, demyelination *in vivo* up-regulates astrocytic IGF-I production and is associated with increased IGF-I receptor expression by oligodendrocytes during myelin regeneration (3, 10, 11).

Other IGF-I actions, such as its rapid reduction in blood–spinal cord barrier permeability, probably also were important in reducing demyelination and promoting clinical recovery (5). In EAE, blood–brain barrier permeability increases before clinical signs and lesions appear and it decreases during recovery (20, 21). IGF-I may act directly on endothelial cells that have IGF-I receptors and can transport IGF-I (22–25). It could reduce permeability by changing the expression of adhesion molecules and other substances that influence lymphocyte and mononuclear cell migration into the central nervous system (26–28).

Effects of IGF-I on immune functions may also produce reduced numbers and areas of demyelinative lesions. In mice, IGF-I administration increases thymus and spleen weights; $CD4^+$ T- and B-lymphocyte numbers increase and immunoglobulin synthesis is enhanced (29, 30). Experiments with cultured human lymphocytes have shown that resting and mitogen-activated T cells have IGF-I receptors that are thought to mediate lymphocyte proliferation and chemotaxis (31). Ongoing studies of IGF-I actions in other EAE models should help clarify how immune functions are changed by treatment. These data also are needed to compare immune-mediated effects of IGF-I with those produced by other agents that have been used to treat or suppress EAE (see, for example, refs. 32–36).

In patients with multiple sclerosis, remyelination has been described in early lesions (37). However, oligodendrocyte functions are reduced as demyelination progresses (4, 38). The blood–brain barrier also is abnormal and there is immune-mediated inflammation (39–41). Although none has been tried to date, growth factors and other agents known to support oligodendrocytes and myelin synthesis are among future strategies described for the treatment of multiple sclerosis (42). Results presented here strongly suggest that IGF-I, at dose levels approximating those already given to patients with endocrine disorders (43), might reduce demyelination, improve oligodendrocyte survival, and promote myelin regeneration in multiple sclerosis.

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